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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(BMS Case No. D0043 NP; MBHB case no. 05-730)

In re Application of:	)	
	)	
Chang et al.	)	Examiner: M. A. Belyavskyi
	)	
Serial No. 10/988,971	)	
	)	Group Art Unit: 1644
Filed: November 20, 2001	)	
	)	
For: Cloning and Expression of Human SLAP-2: A Novel SH2/SH3 Domain-Containing Human SLAP Homologue Having Immune Cell-Specific Expression	)	Confirmation No.: 9658

DECLARATION OF GENA S. WHITNEY

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Gena S. Whitney, hereby declare the following:

1. I received a Bachelor of Science (1982) degree in Cellular and Molecular Biology from the University of Washington, Seattle, WA.

2. Since 1991, I have been an employee at Bristol-Myers Squibb Company, the assignee of this application. My current position is Senior Research Scientist I in the Lawrenceville Discovery Biology/Imunology/Immune Cell Function Group section since 2002. Prior to my employment at BMS, I was employed as a Research associate at the University of Zurich (1986-1988) and at Zymogenetics, Inc. (1982-1986).

3. I have co-authored 25 papers directed, in part, to immunology and signaling mechanisms.

4. A true and accurate copy of my curriculum vitae, which evidences my expertise and credentials, is attached herewith and labeled Exhibit A.

Advisory Notice dated July 8, 2005, the office action dated December 27, 2005, the Applicant's response dated June 27, 2005 and the references cited therein.

6. I have performed an experiment, or directed or caused an experiment to be performed, to provide evidence that hSLAP-2 inhibits anti-human CD3 antibody mediated NFAT promoter activation in Jurkat cell line. My experiment essentially repeats work that was performed and reported by others subsequent to the filing date of this application. See Loreto MP et al. *Mol. Cel. Biol.* 2002 Jun;22(12):4241-55 ("Loreto"); and McGlade CJ et al. *Oncogene* 2003 Jan 16;22(2):266-73 ("McGlade"). The results of this experiment support the original teachings of the subject application that hSLAP-2 is a member of the SLAP family of adapter proteins which function in receptor-ligand signal transduction pathway in cells of the hematopoietic lineage and specifically, that SLAP-2 is a negative regulator of intracellular signal transduction in several cell types, including T-cells. See paragraphs 54 and 76 of the specification. The results of my experiment are also consistent with other findings that show that overexpression of SLAP-2 regulates T cell receptor signaling. See Loreto, McGlade, Pandey et al. *J. Biol Chem.* 2002 May 24;277(21):19131-8. Epub 2002 Mar 12 ("Pandey") and Holland et al. J. Exp. Med. 2001, Vol. 194(9):1263-76.

Materials and Methods:

7. Both the transfection and NFAT luciferase experiments were performed essentially as described in Loreto, McGlade, Liu et al. (*Curr. Biol.*, 1999, Vol. 9:67-75)("Liu") and Berry et al. (*Oncogene* 2001, Vol. 20: 1203-1211)("Berry") except that the Jurkat cells were stably transfected with the NFAT reporter construct and electroporation conditions were modified (300 V, 975 uF). .

8. Cell lines. Jurkat NFAT4 mmx is a Jurkat/ NFAT-promoter reporter cell line constructed with the NFAT promoter linked to the luciferase gene in a pGL3 vector and stably transfected into the Jurkat cell line (Molecumetics, Bellevue, WA) .

9. Cell Culture. The Jurkat/ NFAT promoter-luciferase cell line was maintained in culture media of RPMI Medium 1640 (Gibco Cat. No. 11875-085, Grand Island, NY)/ 10 % FBS (Gibco Cat. No. 16140-071 Grand Island, NY), 1 x Penicillin-Streptomycin (Gibco Cat. No. 15140-122, Grand Island, NY) / 20 mM L-Glutamine (Gibco Cat. No. 25030- 081, Grand Island, NY) and 500 ug/ml Geneticin (Gibco Cat. No.10131-035 Grand Island, NY).

10. Constructs. SLAP-2 _GFP plasmid and the Control_GFP plasmid were constructed by conventional techniques. SLAP-2_GFP included the following insert:

MGSLPSRRKSLPSPSLSSSVQGQGPVTMEAERSKATAVALGSFPAGGPAELSLRLGEPLTIVSEDGDWWTVLSEVSGREYNIPSVHVAK
VSHGWLYEGLSREKAEELLLLPGNPGGAFLIRESQTRRGSYSLSVRLSRPASWDRIRHYRIHCLDNGWLYISPRLTFPSLQALVDHYSE
LADDICCLLKEPCVLQRAGPLPGKPIPLPVTVQRTPLNWKELDSSLLPSEAATGEESLLSEGLRESLSFYISLNDEAVSLDDAKGGRAD
PAFLYKVVDLEGPRFEQKLISEEDLNMHTGHHHHHH. The Control_GFP plasmid contains an arbitrary gene insert for control purposes. Both are in a GFP vector, a pcDNA base with a Gateway cassette, myc and His tag epitopes, IRES element and eGFP sequence. The eGFP was used to assess transfection efficiency.

11. Transient transfections. Exponentially growing Jurkat/ NFAT promoter -luciferase cells were spun down and resuspended in RPMI Medium1640 at 1×10^7 cells/ml in aliquots of 800 µl. Each aliquot of cells received 0, 20 µg or 40 µg of SLAP-2_GFP plasmid or Control_GFP plasmid. The cell/ DNA mixture was incubated at room temperature for ten minutes, transferred to a 0.4 cm Gene Pulser Cuvette (Bio-Rad cat. No. 165-2088, Hercules, CA) and then electroporated (300 V, 975 µF) in a Gene Pulsar II (Bio-Rad, Hercules, CA), following manufacturer's instructions. The electroporated cell/DNA mixture was placed on ice for ten minutes and then transferred to a T25 flask containing 10 ml of culture media. Cells were incubated for 40 hours at 37° C., 5% CO_2.

12. NFAT promoter-luciferase assays. At 40 hours after transfection, cells were centrifuged and resuspended in assay media (RPMI Medium 1640 without phenol red (Gibco Cat. No.11835-030, Grand Island, NY) / 10 % Charcoal/Dextran treated- FBS (HyClone Cat. No. SH30068.03) / 1 x Penicillin-Streptomycin (Gibco Cat. No. 15140-122)/ 20 mM L-Gln (Gibco Cat. No. 25030- 081, Grand Island, NY). Six replicates of each transfection were plated in 96- well assay plates (CulturePlate-96, PerkinElmer Cat. No. 6005680, Boston, MA) at 40,000 viable cells/ well in a 100 µl volume. The cells were either left unstimulated or stimulated with 0.5 µg/ml final concentration of anti- human CD3 antibody, clone G19-4 (Bristol-Myers Squibb, Princeton, NJ), and incubated at 37° C. , 5% CO_2. After six hours, 20 µl of Steady-Glo ® substrate from the Luciferase Assay System (Promega Cat. No. E2510, Madison WI) was added to each well and the lucifease activity was measured with a TopCount NXT (Packard/ PerkinElmer, Boston, MA). See Liu and Berry.

13. Flow cytometry. Assessment of transfection efficiency by FACs analysis. About 1×10^6 cells were harvested and resuspended in phosphate-buffered saline (PBS)/ and then analysed using a FACScan (BD, San Jose, CA). GFP fluorescence was excited at 488 nm and emission was

measured with a 530/30 nm bandpass filter. The gate was set on live cells. Histograms for green fluorescent protein –positive cells were created by using CellQuest software.

Results

14. To determine the effect of SLAP-2 on T cell receptor signaling, NFAT activation was measured using a NFAT promoter-luciferase reporter system. Jurkat/ NFAT promoter -luciferase cells was transiently transfected with 20 µg and 40 µg of SLAP-2_GFP or a control_GFP DNA and its effect was examined on luciferase activity 40 hours post-transfection and after stimulation with anti- human CD3 antibody for six hours. Transfection efficiency was measured by FACs analysis (Figure 1). The results are consistent with other findings (see Holland, Pandey, Loreto, and McGlade) that show that transfection of 40 µg of SLAP-2 DNA into a T- cell NFAT promoter-luciferase reporter cell system significantly inhibits anti- CD3 –induced NFAT promoter activation with a p- value of 1×10^{10}. Transfection with 20 ug of SLAP-2_GFP DNA did not show a significant amount of inhibition when compared to the control.

Conclusions

15. Activation of anti-CD3 triggers an intracellular signaling cascade that leads to the activation of specific nuclear transcription factors, including NFAT. As shown in Figure 2, SLAP-2 inhibits anti- human CD3 antibody mediated NFAT promoter activation in a Jurkat cell line. These results are consistent with other findings that show that overexpression of SLAP-2 negatively regulates T cell receptor signaling. See, for instance, Holland, Pandey, Loreto, and McGlade.

16. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Gena Saari Whitney

Gena Saari Whitney

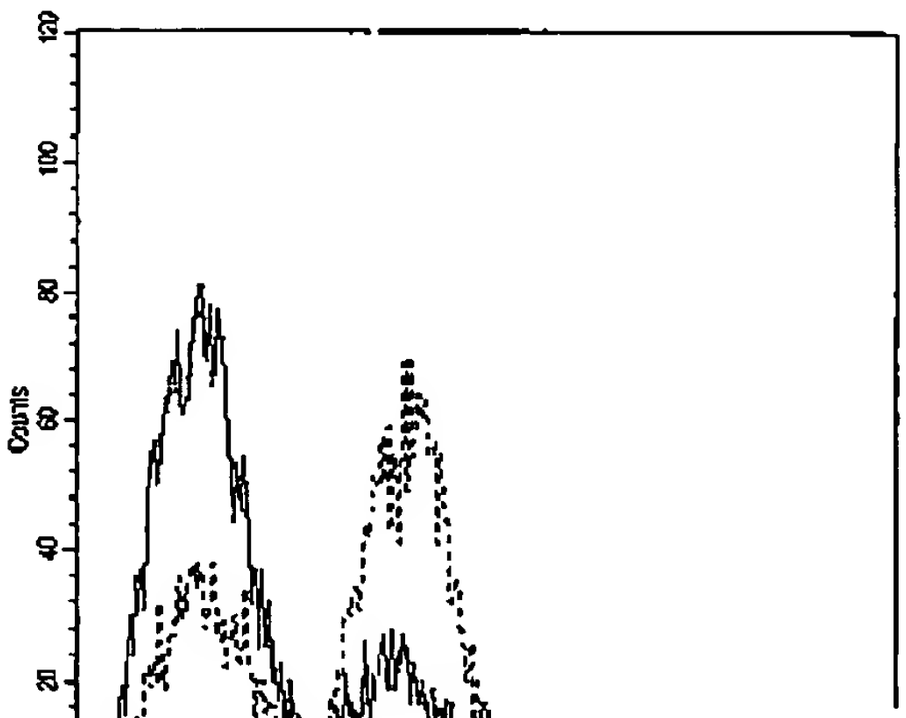
Feb 15, 2006

Date

Enclosures: Figures 1, 2 and Exhibit A

Figure 1. *Assessment of transfection efficiency by FACs analysis.* Histograms for green fluorescent protein –positive cells were created by using CellQuest software. On the histograms, cells transfected with no DNA are shown with a solid line and transfected cells, Control_GFP (a) and SLAP-2_GFP (b) are shown as an overlays with dotted lines.

a.



b.

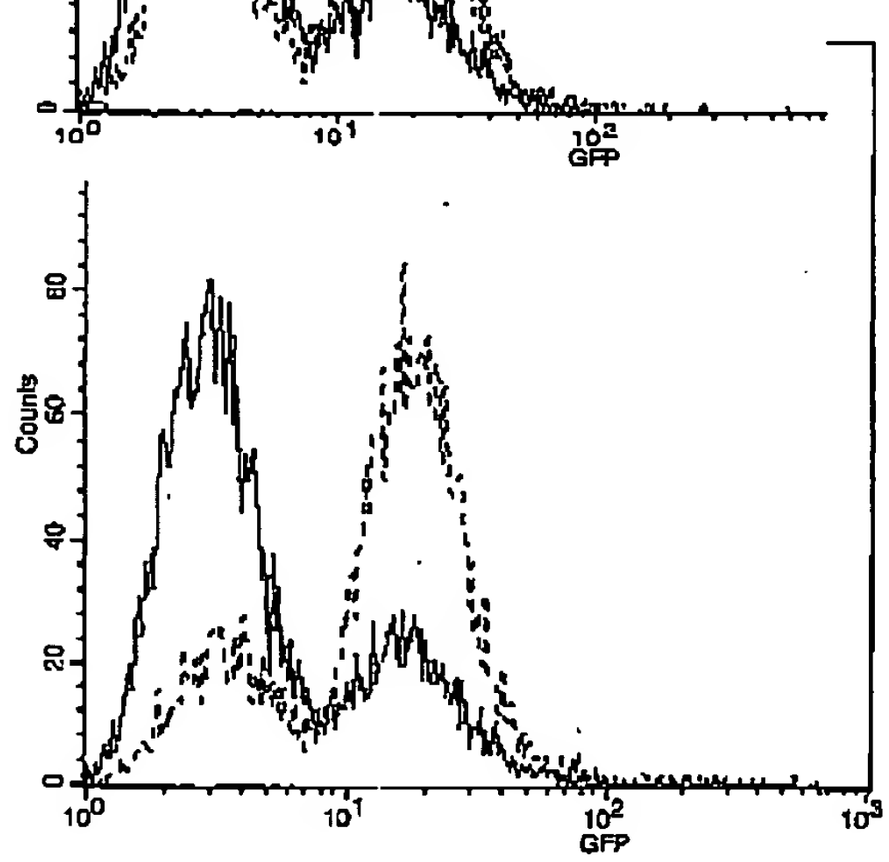


Figure 2. *NFAT Promoter Activation is inhibited by SLAP-2.* NFAT promoter -luciferase Jurkat cells were transiently transfected with no DNA, 40 µg of a Control_GFP plasmid or 40 µg of SLAP-2_GFP plasmid by electroporation (300 V, 975 µF). After 40 hours, the cells were centrifuged and resuspended in assay media. Six replicates of each transfection of 40,000 viable cells/ well in a 100 µl volume were plated in 96- well assay plate. Half of the cells were incubated for six hours with anti-human CD3 antibody (+) and the other half wre left untreated (-). After six hours stimulation, luciferase activity was assayed. Error bars indicate standard deviations.